IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Brakel et al.	Group Art Unit: 1631
Serial No.:	08/479, 999	Examiner: J. Zhou
Filed:	June 28, 1994	Confirmation No.: 8801
For: MODIFIED NUCLEOTIDE COMPOUNDS		

RESPONSE TO NOTIFICATION OF NONCOMPLIANT APPEAL BRIEF

Mail Stop Appeal
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Notification of Noncompliance dated November 29, 2007, Appellants note that in the previous Appeal Brief submitted in December 2006, the status of claims and status of amendments were provided. However, Appellants herewith submit an Appeal Brief where Appellants have provided a table indicating the status of all of the claims in the application and a table indicating the status of amendments. Claims 20, 39, 40, 44 and 49 are pending. Claims 20, 39 and 40 have been allowed. Thus, only claims 44 and 49 are on Appeal.

Appellants also submit an accompanying Amendment Under 37 C.F.R. §1.116.

Respectfully submitted,

Cheryl H. Agris

Dated: 12/29/07

Cheryl H. Agris, Reg. No. 34,086
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Enz-47(C)(2)

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527 Madison Avenue (9th Floor)
New York, New York 10022

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APPEAL BRIEF

I. REAL PARTY IN INTEREST

The real party in interest of the present application is Enzo Therapeutics, Inc., which is a subsidiary of Enzo Biochem., Inc. (hereinafter "Enzo"). Enzo is the owner of the present application by way of an assignment from the inventors, Brakel et al., of all rights, title, and interest.

II. RELATED APPEALS AND INTERFERENCES

There are no appeals or interferences related to the present application.

III. STATUS OF CLAIMS

As noted in the response to the Notice to Comply, Appellants had previously indicated the status of the claims. However, in order to be completely responsive to

Remarks made by the Examiner in the Notice to Comply and in order to clarify the situation, the status of each of the claims are identified in the table below.

Claim	Status
1	Canceled
2	Canceled
3	Canceled
4	Canceled
5	Canceled
6	Canceled
7	Canceled
8	Canceled
9	Canceled
10	Canceled
11	Canceled
12	Canceled
13	Canceled
14	Canceled
15	Canceled
16	Canceled
17	Canceled
18	Canceled
19	Canceled
20	**Allowed**
21	Canceled

Claim	**Status**
22	**Canceled**
23	Canceled
24	Canceled
25	Canceled
26	Canceled
27	Canceled
28	Canceled
29	Canceled
30	Canceled
31	Canceled
32	Canceled
33	Canceled
34	Canceled
35	Canceled
36	Canceled
37	Canceled
38	Canceled
39	**Allowed**
40	**Allowed**
41	Canceled
42	Canceled
43	Canceled
44	**Rejected On Appeal**

Claim	Status
45	Canceled
46	Canceled
47	Canceled
48	Canceled
49	**Rejected** **On Appeal**
50	Canceled
51	Canceled
52	Canceled

As indicated in the table above, claims 44 and 49 are on appeal; no other claims are on appeal. The other pending claims, claims 20, 39 and 40 have been allowed.

IV.STATUS OF AMENDMENTS

As noted in the Response to the Notice to Comply, Appellants did indicate the **Status of ALL Amendments** submitted subsequent to the Final Rejection in the Appeal Brief submitted in December 2006. It is reproduced below.

> After the Final Office Action dated September 26, 2000, the following were submitted by Appellants: a first amendment under 37 C.F.R. §1.116 in response to the Office Action dated September 26, 2000 and a second amendment under 37 C.F.R. §1.116 was submitted with the Appeal Brief filed on July 19, 2004 and November 29, 2004. Advisory actions were issued in response to these amendments. Neither of these amendments were entered.
>
> A third amendment under 37 C.F.R. §1.116 was submitted with the Appeal Brief filed on February 14, 2006. An Advisory action was issued in response to this third amendment indicating that the amendment had been entered.

However, to to be completely responsive and provide a complete updated status report the status of all of the amendments submitted after the Final Rejection dated September 26, 2000 are provided in the table below

Date Amendment Submitted	**Nature of Amendment**	**Entered**
December 26, 2001	Claims 1, 18, 19, 21, 37, 41 and 51 were amended; claim 52 was canceled	No
July 19, 2004	Claims 1, 21, 40 and 50 were amended Claims 53 and 54 were added	No
February 14, 2006	Claims 1-19, 21-48, 41, 51 and 52 were canceled Claim 39 was amended	Yes
December 8, 2006	Claims 44 and 49 were canceled	No

V. SUMMARY OF INVENTION

The present invention is directed to modified nucleotide compounds complementary to at least a portion of and effective to inhibit the function of an RNA of an organism when administered. These compounds are resistant to nucleases yet form an RNase H substrate when hybridized to a complementary RNA sequence. The invention is also directed to methods of inhibiting the function of RNA using said compounds and a method of identifying a nucleotide compound having a combination of nuclease resistance and ability to form an RNase H substrate when in complex

with an RNA. The next three pages show a table showing the support in the specification for each of the claims.

Claim	Claim Elements	Support in the Specification
Modified nucleotide compound Claim 20	contains at least one sequence having the formula MN_3M ; N is a phosphodiester-linked unmodified 2'-deoxynucleoside moiety containing at least one guanine, adenine, cytosine or thymine moiety; M is a methylphosphonate-containing deoxynucleotide	Page 8, lines 13-19 and original claim 20 (page 23, bottom 2 lines and page 24, line 3).
Method of inhibiting function of an RNA wherein RNA is contacted with a compound Claim 39	Modified nucleotide compound includes at least one sequence having the formula MN_3M wherein N is a phosphodiester-linked unmodified 2'-deoxynucleoside moiety containing at least one guanine, adenine, cytosine or thymine moiety and M is a methylphosphonate-containing deoxynucleoside	Page 8, lines 17-26; original claim 39 (page 25, lines 7-12)

Claim	Claim Elements	Support in the Specification
Method of identifying a nucleotide compound Claim 40	Compound has a combination of nuclease resistance and the ability to form an RNase H substrate when in complex with an RNA. Method comprises: (i)preparing modified nucleotide compounds; (ii)selecting by exo-and endonuclease digestion those modified nucleotide compounds (i) which are nuclease-resistant as shown by being capable of forming and electrophoretically migrating as a duplex with a complementary nucleotide compound; and selecting by RNase H digestion nuclease-resistance nucleotide compounds of (ii) which act as substrates for RNase H when hybridized with a complementary RNA	Paragraph bridging page 8 and 9; original claim 40 (page 26, lines 15-25)

Claim		Claim Elements	Support in the Specification
Compound containing at least 2 separate nuclease resistant components (claim 44 and 49)	44	Confers RNase H sensitivity upon the RNA when complexed with a complementary RNA	See example 4 (page 18, line 1 to bottom of page 19)
	49	Portion of the compound that can function as an RNase H substrate is located between the moiety conferring exonuclease resistance and the moiety conferring endonuclease resistance	See Examples, particularly (see Table 1, page 11 and page 19, lines 28-33)

VI. ISSUES

There is one issue on appeal, whether claims 44 and 49 are unpatentable over 35 U.S.C. §102(b) over Miller et al., 1985, Biochimie 67:769-776 (hereinafter "Miller et al., 1985").

VII. GROUPING OF CLAIMS

Appellants submit that the claims on appeal, claims 44 and 49 stand and fall together.

VIII. ARGUMENT

The present invention is directed to novel modified nucleotide compounds that contain endo and exonuclease resistant components and can form RNase H substrates when complexed with a complementary RNA as well as a method for identifying such compounds. The invention is also directed to methods of using these compounds, specifically, inhibiting the function of an RNA and treating a human or animal so as to inhibit the function of a target RNA as well as methods for identifying such compounds.

As will be discussed in further detail below, the claimed subject matter is not anticipated by Miller et al., 1985. Each of the currently remaining rejections are addressed below.

The originally pending claims 1, 2, 4, 8, 12-14, 19 and 42-50 were rejected under 35 U.S.C. §102(b) as being anticipated by Miller et al., 1985. As noted above, claims 1-19, 21-38, 41-43, 45-48, and 50-52 have been canceled.

Claim 44 recites that the claimed compound acts as an RNase H substrate when complexed with complementary RNA and that each nuclease resistant component comprises at least one moiety which confers endonuclease resistance and at least one moiety which confers exonuclease resistance and that 2 or more contiguous phosphodiester-linked 2' deoxynucleosides are located between the moiety conferring endonuclease resistance and the moiety conferring exonuclease resistance.

Claim 49 contains the further limitation that the compound additionally contains a modified oligonucleotide or polynucleotide, which consists of at least one moiety which confers endonuclease resistance and at least one moiety which confers exonuclease reisstance. In contrast to the other rejected claims only claims

Appellants further note that the cited Miller et al. reference is silent with respect to the RNase H sensitivity of the methylphosphonate sequences disclosed in Figure 3. However, it is most likely that these sequences are RNase H resistant. For example, Cazenave et al., 1989, Nucl. Acids Res. 17:4255-4273 (**Tab 1** and cited in the instant application) discloses that a methyl phosphonate 17-mer "failed to induce the degradation of the target mRNA by the E. coli RNase H". Additionally, Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204 (**Tab 2**) discloses results from studies with a 14-mer oligonucleotide containing one to six methylphosphonate linkages. Results from the Furdon et al. studies indicated that "Susceptibility to cleavage by RNase H increased parallel to a reduction in the number of methylphosphonate residues in the

oligonucleotide"[1]. It is further noted in Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204[2]

> RNA hybridized to MP-oligos containing one or two methylphosphonate deoxynucleosides was cleaved by RNase H almost a easily as that in the control duplex with D-oligo,,,,RNA in duplexes with MP-oligos which contained three, four and six methylphosphonate deoxynucleosides, i.e., in which methylphosphonate bonds were separated by three, two or one phosphodiester bond...was increasingly resistant to cleavage by the enzyme.

Although it is Appellants position that claims 44 and 49 are not anticipated by Miller et al., Appellants in order to advance prosecution are concurrently submitting a Fourth Amendment under 37 C.F.R. §1.116 where claims 44 and 49 have been canceled.

IX. CONCLUSION

Claims 44 and 49 are patentable over Miller et al. Claims 44 and 49 specifically recite that the claimed compound when complexed with a complementary RNA confers RNase H sensitivity upon the RNA. However, in order to advance prosecution, claims 44 and 49 have been canceled in the accompanying amendment under 37 CFR 1.116.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

Respectfully submitted,

Cheryl H. Agris

Dated: 12/29/07

Cheryl H. Agris, Reg. No. 34,086
Attorney for Appellants

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[1] See abstract in Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204
[2] See Furdon et al. at page 9202

APPENDIX A

APPENDIX A-CLAIMS ON APPEAL

What is Claimed Is:

44 A compound containing at least 2 separate nuclease resistant components each consisting of 2 or more contiguous phosphodiester-linked 2' deoxynucleosides; wherein at least one of said contiguous phosphodiester-linked 2' deoxynucleosides is unmodified,, when complexed with a complementary RNA, confers RNase H sensitivity upon the RNA.

49. A compound containing at least 2 separate nuclease resistant components each consisting of 2 or more contiguous phosphodiester-linked 2' deoxynucleosides; wherein at least one of said contiguous phosphodiester-linked 2' deoxynucleosides is unmodified and wherein said compound further comprises a modified oligonucleotide or polynucleotide, wherein the modified oligonucleotide or polynucleotide consists of at least one moiety which confers endonuclease resistance and at least one moiety which confers exonuclease resistance, wherein the portion of the compound that can function as an RNase H substrate is located between the moiety conferring exonuclease resistance and the moiety conferring endonuclease resistance.

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Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides

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ABSTRACT

We have studied the translation of rabbit globin mRNA in cell free systems (reticulocyte lysate and wheat germ extract) and in microinjected *Xenopus* oocytes in the presence of anti-sense oligodeoxynucleotides. Results obtained with the unmodified all-oxygen compounds were compared with those obtained when phosphorothioate or α-DNA was used. In the wheat germ system a 17-mer sequence targeted to the coding region of β-globin mRNA was specifically inhibitory when either the unmodified phosphodiester oligonucleotide or its phosphorothioate analogue were used. In contrast no effect was observed with the α-oligomer. These results were ascribed to the fact that phosphorothioate oligomers elicit an RNase-H activity comparable to the all-oxygen congeners, while α-DNA/mRNA hybrids were a poor substrate. Microinjected *Xenopus* oocytes followed a similar pattern. The phosphorothioate oligomer was more efficient to prevent translation than the unmodified 17-mer. Inhibition of β-globin synthesis was observed in the nanomolar concentration range. This result can be ascribed to the nuclease resistance of phosphorothioates as compared to natural phosphodiester linkages. α-oligomers were devoid of any inhibitory effect up to 30 μM. Phosphorothioate oligodeoxyribonucleotides were shown to be non-specific inhibitors of protein translation, at concentrations in the micromolar range, in both cell-free systems and oocytes. Non-specific inhibition of translation was dependent on the length of the phosphorothioate oligomer. These non-specific effects were not observed with the unmodified or the α-oligonucleotides.

INTRODUCTION

The use of antisense oligodeoxynucleotides as specific inhibitors of gene expression has undergone a rapid expansion over the past several years (1,2). Central to this approach is the presumption that messenger RNAs bound as RNA-DNA duplexes either cannot be translated by ribosomes (3-5) or are destroyed by RNase-H (6-10). In order to be effective *in vivo*, synthetic oligonucleotides must share several properties. Among these are :1) chemical stability, 2) water solubility, 3) high thermodynamic stability of the RNA-DNA duplex and 4) nuclease resistance. Unmodified phosphodiester (PO) DNA meets these requirements except for the last one. Thus, a series of modified derivatives have recently been synthesized (11-14). Substitution of sulfur for one of the phosphodiester oxygen atoms yields a molecular species that meets all four criteria (12). These phosphorothioate (PS) oligodeoxynucleotides have indeed recently been shown to inhibit the cytopathic effect of HIV-1 (15; Matsukura et al., unpublished results). In experiments in chronically infected H9 cells, the expression of p24 *gag* protein was shown to fall >90% in the presence of a 10 μM concentration of a

phosphorothioate sequence complementary to the 5' region of the *rev* (formerly *art/trs*) gene. The normal congener was ineffective as was a methylphosphonate construct. Marcus-Sekura et al. (16) showed that an anti-sense phosphorothioate was an effective inhibitor of chloramphenicol acetyl transferase activity in the standard CAT assay. In a series of experiments conducted in HL60 cells, an anti-sense c-*myc* phosphorothioate oligomer was unable to consistently inhibit cellular proliferation unless supplied in liposomes, whereas in multiple experiments, the normal oligomer inhibited levels of myc protein by >50% at 12 h (17).

Another class of modified oligonucleotide meets the criterion of nuclease resistance. In these compounds the natural β-configuration of the nucleoside is transposed into its α-analogue (13, 14, 18-26). However these α-oligonucleotides were reported to be poor inhibitors of VSV mRNA translation in rabbit reticulocyte lysate (27).

Cell-free systems (28-34) and micro-injected *Xenopus* oocytes (8,35-37) are effective means for evaluating the ability of modified oligonucleotides to act as antisense inhibitors. We present here a comparative study of both unmodified and nuclease resistant oligonucleotides tested for their ability to promote selective arrest of rabbit globin mRNA translation.

MATERIALS AND METHODS

Oligodeoxynucleotides

Phosphodiester oligodeoxynucleotides were synthesized either on a Pharmacia or on an Applied Biosystems Model 380B Synthesizer, and were purified via high-pressure liquid chromatography (Waters) on a PRP-1 column. Phosphorothioates were synthesized and purified via a modification (12) of the procedure of Stec et al. (38). Alpha-oligodeoxynucleotides were synthesized on a Pharmacia automatic synthesizer and purified as previously described (24). The oligomer length homogeneity was periodically evaluated by running samples on 15% polyacrylamide/6M urea gels. After electrophoresis, bands were either stained with ethidium bromide and viewed by UV-light or revealed by autoradiography in the case of ^{32}P-labelled oligonucleotides. All preparations yielded a single species in each lane loaded.

Cell-free translation systems

Wheat germ extract was purchased either from New England Nuclear or from Genofit (Geneva). Oligomer was added to a translation mixture containing ^{35}S-methionine. Unless otherwise stated experiments in wheat germ extracts were performed under the following conditions: 0.05 µg of rabbit globin mRNA was mixed with the oligonucleotide and added to 30 µl of the translation mixture. The final concentration of total mRNA was 9.3nM (i.e. 3.9nM in β-globin). The reaction was run at 25°C during 30 min. Reactions were generally carried out without premixing RNA with the oligomer. We showed that premixing did not alter the results.

Rabbit reticulocyte lysate was purchased from New England Nuclear. The oligomer, at

the appropriate concentration, was added to the translation mixture (25µl) containing 0.1µg globin mRNA and ^{35}S-methionine. The samples were then incubated for 90 min at 37°C.

An aliquot of the reaction mixtures was then analyzed either on a 15% polyacrylamide-SDS gel with a 5% stack or on a 12% polyacrylamide gel containing 8 mM Triton X100 and 6M urea. The gels were then fixed in a 40% methanol/7% acetic acid solution for about 1h, soaked in a solution of sodium salicylate ("Fluoro-Hance", Research Products International Corp.,) for 30 min, and dried under vacuum prior to autoradiography.

Translation in *Xenopus* oocytes

Stage 6 oocytes (selected via stereotactic microscopy) were obtained from the Laboratoire de Physiologie de la Reproduction (Paris VI University). Specimens were maintained in modified Barth's saline solution (39). 80 nl of a 1/1 (v/v) mixture of globin mRNA (50 µg/ml) and oligomer, dissolved in sterile distilled water, were injected in *Xenopus* oocytes; 4 to 5 hours after injection, oocytes were incubated in the presence of ^{35}S-methionine for about 15 hours. The samples were then homogenized in 20 µl (per oocyte) of 20 mM Tris, pH 7.6, 0.1 M NaCl, 1% Triton X100 and 1 mM PMSF (40). Proteins were then analysed by SDS-PAGE electrophoresis on a 13.5% acrylamide gel. Assuming a free diffusion compartment of 0.5 µl inside the oocyte, the final intracellular concentration of β-globin mRNA was about 16 nM.

Hybridization studies

1 µg of rabbit globin mRNA was bound to a nitrocellulose filter by heating at 80°C during 2 hours. The filter was incubated in a mixure containing about 10^7 cpm of ^{32}P-labelled oligomer and 2 ml of 6xSSC/10xDenhardt's solution (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2; 10xDenhardt's is 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl-pyrolidone). The filters were then placed in a thermostated holder, and were eluted with 6xSSC as the temperature was increased at a rate of 1.2°C/min. Thermal elution profiles were constructed, and the Tc determined to be that temperature at which 50% of the total counts had been eluted (41).

Oligodeoxynucleotide-promoted cleavage of globin mRNA by RNase-H

RNase-H from *E. coli* was obtained from Genofit. Kinetic experiments were run at 37°C in a 20 mM Tris-HCl buffer, pH 7.5 containing 100 mM KCl, 10 mM $MgCl_2$ and 0.1 mM dithiothreitol. Incubations of 0.3µg of rabbit globin mRNA and 30pmoles of oligonucleotide were performed in a total volume of 30 µl, in the presence of 2.5 units of enzyme. At the appropriate times aliquots of 5 µl were spotted on a nylon membrane. UV-irradiated membranes were probed with ^{32}P 5' end-labelled 17PO-β (Figure 1) and autoradiographed.

RESULTS

Sequences studied

The sequences of the oligonucleotides used throughout this study and of the complementary regions on rabbit α- and β-globin mRNAs are given in figure 1. We targeted

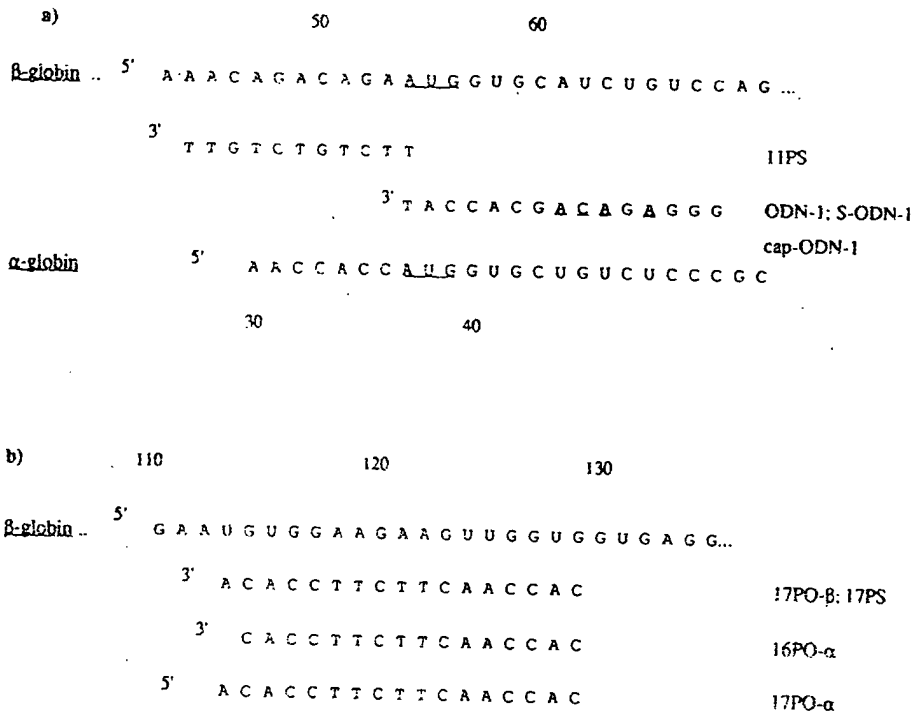


Figure 1 : Nucleotide sequences of oligodeoxynucleotides complementary to a) AUG region of rabbit β-globin and α-globin (51) and b) coding sequence of rabbit β-globin mRNA. The numbering above the RNA sequences refer to the transcription start; the translation initiation codons are underlined. The abbreviations of the antisense oligonucleotides are indicated on the right of the sequences (for details see "Results; Sequences studied"). 11-mers, 17-mers and the 16-mer were complementary to the β-globin mRNA and ODN-1 to the α-globin mRNAs. The latter oligonucleotide can pair with the β-message giving 4 mismatches (underlined letters in the ODN-1 sequence).

two regions of the β-globin mRNA, namely nucleotides 44-54 and 113-129 that were already selected in a previous study (8). The 44-54 sequence is located immediately upstream of the start codon, while the 113-129 sequence is within the coding region of the message. An unmodified phosphodiester (PO) oligonucleotide (17PO-β) and phosphorothioate (PS) analogues (11PS; 17PS), complementary to these two regions, were synthesized. Two α-oligodeoxynucleotides complementary to the coding region were also used. The first one, 16PO-α, was designed to be in an antiparallel orientation with respect to the target sequence. A second one, 17PO-α was synthesized to bind its target in a parallel orientation.

In addition we constructed several oligomers complementary to the α-globin mRNA sequence. ODN-1 is a 15-mer sequence complementary to the α-globin initiation codon and downstream region (Figure 1). This sequence also complements a similar region of the β-globin message with the exception of a 4-base mismatch, assuming the formation of two G-U

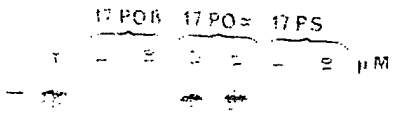


Figure 2 : Effect of various 17-mers on in vitro synthesis of rabbit β-globin. Autoradiograph of a 12% Triton-acetic acid-urea polyacrylamide gel of proteins synthesized in wheat germ extracts as indicated in Materials and Methods in the absence (T) or in the presence of 17PO-β, 17PO-α or 17PS at the indicated concentration. The upper band (arrow) corresponds to β-globin and the lower one to α-globin.

pairs. ODN-1[Sen] is the sense construct. ODN-1 and ODN-1[Sen] were also synthesized in all-phosphorothioate forms (S-ODN-1; S-ODN-1[Sen]). In addition, they were also constructed with two phosphorothioates at both the 3' and 5' ends, and are referred to as cap-ODN-1 and cap-ODN-1[Sen], respectively.

A series of random sequences were also made. ODN-2 (5'-dACTCC-3') is a 5-mer, and ODN-3 (5'-dCCAAACCATG-3') a 10-mer. ODN-2 and -3 were synthesized as phosphorothioate derivatives only. A random 16-mer, termed ODN-4, with base composition equivalent to ODN-1 (5'-dACGCGAGGACCATAGT-3') contained 5 contiguous phosphorothioates at the 5' and 3' ends, separated by 5 phosphodiester linkages. We also synthesized the same 16-mer (5'-dACGCGAGGACCATAGT-3') containing alternating PS and PO linkages (ODN-5).The absence of perfect complementarity between these oligomers and rabbit globin mRNAs was checked by a computer search (CITI 2).

Table 1

Oligos	Tc	WGE	*Xenopus* oocytes		
			oligo/RNA	Coinjection	RNA/oligo
17PO-β	60	50	>>	30	650
17PO-α	50	>>		>>	
17PS	39	50	30	3	7
11PS	28	3000		8000	

Table 1 : Stability of oligonucleotide/RNA hybrids and inhibition of translation by antisense oligonucleotides. Temperature of half-dissociation (Tc) of filter-bound complexes, determined as described in Material and Methods are given in °C. Concentrations (indicated in nM), leading to a 50% decrease of translation either in wheat germ extracts (WGE) or in oocytes following co-injection or delayed injections (RNA/oligo means RNA first; oligo/RNA means oligo first; see text) were determined from curves shown on figures 3 and 6. The symbol >> indicates that 50% inhibition was not reached at the highest concentration tested.

Effects of oligonucleotides on translation in cell-free media

Translation in wheat germ extract : Translation of rabbit globin mRNA in cell-free systems gives rise to two bands corresponding to the α- and β-chains which can be separated on Triton-urea-acetic acid polyacrylamide gels. In a first set of experiments we compared the effects on globin synthesis of three 17-mers, 17PO-β, 17PO-α and 17PS, targeted to the coding region of β-globin mRNA (Figure 1). The results shown in figure 2 indicate that 17PO-β is a specific anti-sense inhibitor in a wheat germ system: a fifty per cent decrease was observed at 50 nM (Table 1) and a total inhibition of β-globin synthesis was attained at 1 μM, whereas α-globin synthesis was not affected, in good agreement with a previous report (8). In contrast even at 11 μM no effect was observed in the presence of 17PO-α. At low concentration (below ca. 1 μM) a specific decrease of β-globin synthesis resulted from addition of 17PS to the translation mixture, 50% inhibition being observed at 50 nM as in the case of 17PO-β (Table 1). However, by 1-2 μM, the synthesis of α-globin was also decreasing and at 10 μM 17PS both α- and β-globin mRNA synthesis were 100% inhibited (Figure 2). As this oligonucleotide is not complementary to any region of α-globin mRNA (no match above than 70% homology) this should be ascribed to a non-specific effect on translation. Thus, in a defined concentration range (<500nM), the anti-sense 17PS inhibits the synthesis only of its directed target, i.e., β-globin; and the sense analogue, in that same concentration range, has little if any effect on the synthesis of either α- or β-globin (data not shown).

A similar conclusion regarding antisense specificity can be drawn from experiments with 11PS, a phosphorothioate oligomer complementary to the region immediately upstream of the AUG codon of β-globin (Figure 1), homologous to an unmodified 11-mer (11PO) that we used in a previous study (8). Specific inhibition of β-globin synthesis (at low concentration) and non-specific inhibition of α-globin mRNA translation (at high concentration) were observed but 11PS was a less efficient inhibitor than 11PO. It should also be noted that higher concentrations of 11PS (>5 μM) than those of 17PS were required to observe non-specific effects (Figure 3). This can be related to a length effect (see below).

We also tested ODN-1, a 15-mer complementary to the α-message (Figure 1), in the wheat germ system; 100% inhibition of translation was achieved at 1 μM (Figure 4) but no inhibition was observed with the sense congener (data not shown). On the other hand, both sense and anti-sense S-ODN-1 constructs were potent inhibitors of translation at 5 μM, with control levels being reached at ca. 500 nM (Figure 4). The region from 500 nM to 5 μM was not examined in greater detail in this study. Translation of Brome Mosaic Virus mRNA was also inhibited, in the wheat germ system, in the presence of S-ODN-1[Sen], to which it has no sequence homology above 70% (Figure 4).

We evaluated the dependence of non-specific translation inhibition on phosphorothioate oligomer length. The random 5-mer ODN-2 was not inhibitory to globin mRNA translation up to 25 μM and only partially inhibitory at higher concentrations (Figure 4). If the random

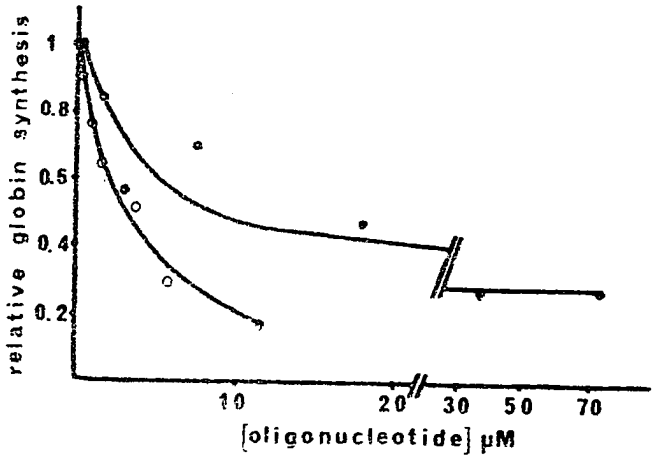


Figure 3 : Effect of 11PS on rabbit β-globin synthesis. Globin mRNA was either translated in wheat germ extracts (O) or in micro-injected *Xenopus* oocytes (●). ^{35}S-labelled proteins were analyzed by gel electrophoresis (see Materials and Methods). β-globin synthesis was determined from densitometer tracings of autoradiographs, relatively to the synthesis observed in the absence of added oligodeoxynucleotide.

10-mer, (ODN-3), was used in the translation assay, control levels of protein synthesis were not achieved at concentrations higher than 3 µM. ODN-3 has no complementary sequence matching better than 70%, neither in α- nor in β-globin mRNA. In the case of ODN-1 several sequences where found that could form four base pairs. Such hybrids are not expected to be stable under our conditions. A 28-mer, S-dC_{28}, was also examined in the wheat germ system. This oligomer is capable of inhibiting the cytopathic effect of the HIV virus in newly infected H9 cells, at concentrations in the low micromolar range (15). S-dC_{28} was the most potent inhibitor tested in this system: translation of globin mRNA was entirely inhibited above 500 nM.

As the non-specific inhibitory properties of phosphorothioate oligodeoxynucleotides appeared to be highly length dependent, we wanted to determine if a molecule containing blocks of contiguous phosphorothioates (where each block was itself too short to be an inhibitor) could act in summation to produce an effective translation inhibitor. Because we knew that a 5-mer phosphorothioate had a limited inhibitory effect on translation, we synthesized a random 16-mer ODN-4, with base composition essentially equivalent to ODN-1, containing 5 contiguous phosphorothioates at the 5' and 3' ends separated by 5 phosphodiester linkages. When tested in the wheat germ system, 100% inhibition of translation was seen at concentrations higher than 6.25 µM (Figure 4). Thus, this molecule does not behave as if it were two separated 5-mers, but rather as if it were a 10-mer (compare to ODN-3).

The non-specific inhibition of translation is not related only to the length of the

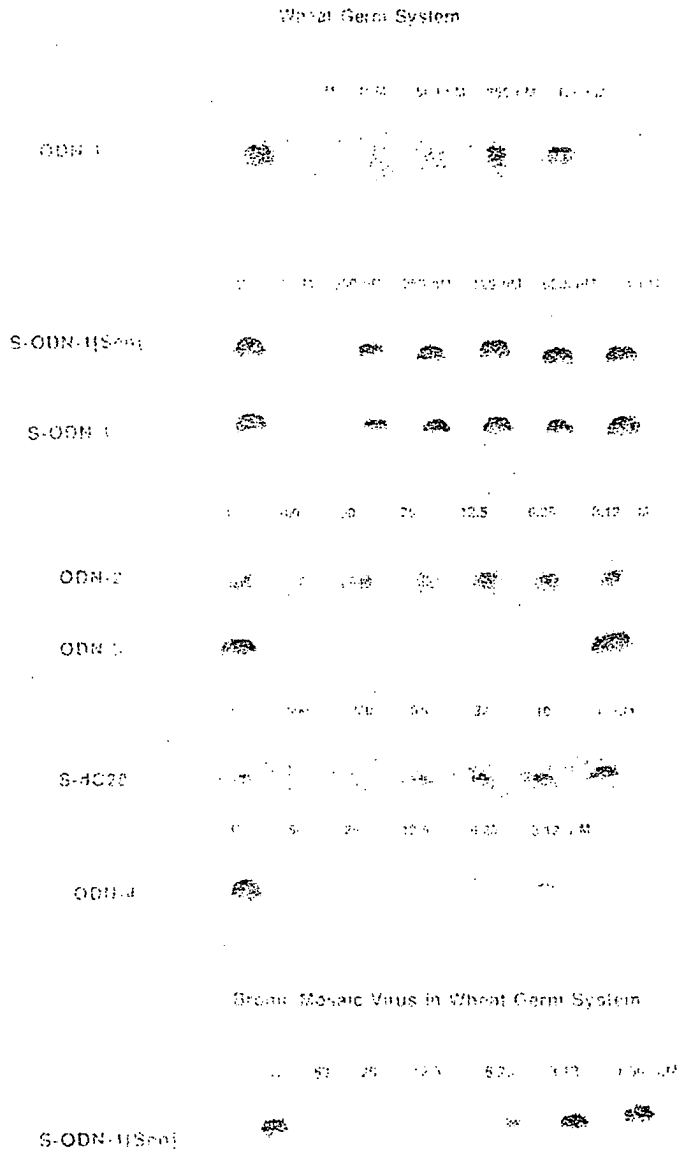


Figure 4 : Translation of rabbit globin mRNA and Brome Mosaic Virus mRNA in the wheat germ system in the presence of various oligomers: ODN-1 is a 15-mer complementary to α-globin mRNA; S-ODN-1 is the phosphorothioate analogue; ODN-1[Sen] and S-ODN-1[Sen] are the sens oligomers; cap-ODN-1 contains two phosphorothioate linkages at the 5'- and at the 3'-ends; ODN-2 and ODN-3 are random phosphorothioate 5-mer and 10-mer, respectively; ODN-4 is a random 15-mer composed of two blocks of 5 phosphorothoates separated by 5 phosphodiesters (for more details see "Results; Sequences studied"). C=control (no added oligomer). Numerals above each lane are the concentration of added oligomer (in μM or in nM). Incubation time was 120 min. at 22°C.

oligomer: cap-ODN-1 (antisense) also inhibited protein translation (100% below 3 μM) while the sense congener exhibited dose dependent inhibition above 3 μM (data not shown). This non-specific inhibition was unexpected as the molecule contained only two phosphorothioates at each end. In contrast, a concentration of 100 μM ODN-5, a random 16-mer with essentially the same base composition as ODN-1 and containing eight alternating phosphorothioates, was required for 100% inhibition of translation (data not shown).

Translation in rabbit reticulocyte lysate : When ODN-1 was used in the reticulocyte lysate system, concentration dependent inhibition of globin synthesis was observed, with 100% inhibition (α- plus β-) seen at 100 μM, while no inhibition was seen for the sense analog (5'-dATGGTGCTGTCTCCC-3') up to 100 μM (Figure 5). This is approximately a 100-fold decrease in sensitivity as compared to the wheat germ extract, and may reflect low levels of RNase-H activity present in reticulocyte lysate (7). However, when phosphorothioates of identical sequence were used under similar reaction conditions, both the sense and the antisense 15-mers were 100% inhibitory (α- plus β-globin) above 6.25 μM (Figure 5). Note that the sense construct appears to be even more inhibitory than the anti-sense species below 6.25 μM. This observation precludes the existence, under these reaction conditions, of even a narrow concentration range, or "window", of anti-sense specificity in the rabbit reticulocyte system.

The effect of S-oligomer length on inhibition of translation was also evaluated in the reticulocyte lysate system. ODN-2, a random 5-mer, was not inhibitory at any concentration tested (up to 100 μM), while ODN-3, the random 10-mer, was inhibitory (α- plus β-globin) at concentrations above 25 μM. S-dC_{28} and its phosphodiester congener O-dC_{28} were tested. They both bind to the reverse transcriptase of HIV-1 (50) but O-dC_{28} has a lower affinity than S-dC_{28}. In the reticulocyte lysate system, O-dC_{28} did not inhibit protein translation at 100 μM, while S-dC_{28} was virtually completely inhibitory at concentrations higher than 3 μM (Figure 5).

Variants of ODN-1, which contains two phosphorothioates only at the 3' and 5' end, were also examined in the reticulocyte lysate system. For the antisense construct (cap-ODN-1), a dose dependent inhibition of protein translation was observed, with 100% inhibition (α- plus β-globin) seen at 100 μM (Figure 5). Control levels of translation were achieved below 25 μM. In experiments with the sense construct (cap-ODN-1[Sen]), no inhibition was seen until a concentration of 100 μM was obtained. Thus, in this system, there appears to be a window of antisense specificity in the 25-100 μM concentration range.

Xenopus oocytes :

Following micro-injection, β-globin mRNA is efficiently translated in *Xenopus* oocytes (the synthesis of the α-polypeptide requires the presence of hemin). Figure 6 displays the results of the gel electrophoresis of oocyte proteins after co-injection of rabbit globin mRNA with two different concentrations of both normal (17PO-β), α (17PO-α) and phosphorothioate (17PS) oligomers. Results similar to those in the wheat germ system were obtained. 17PO-α

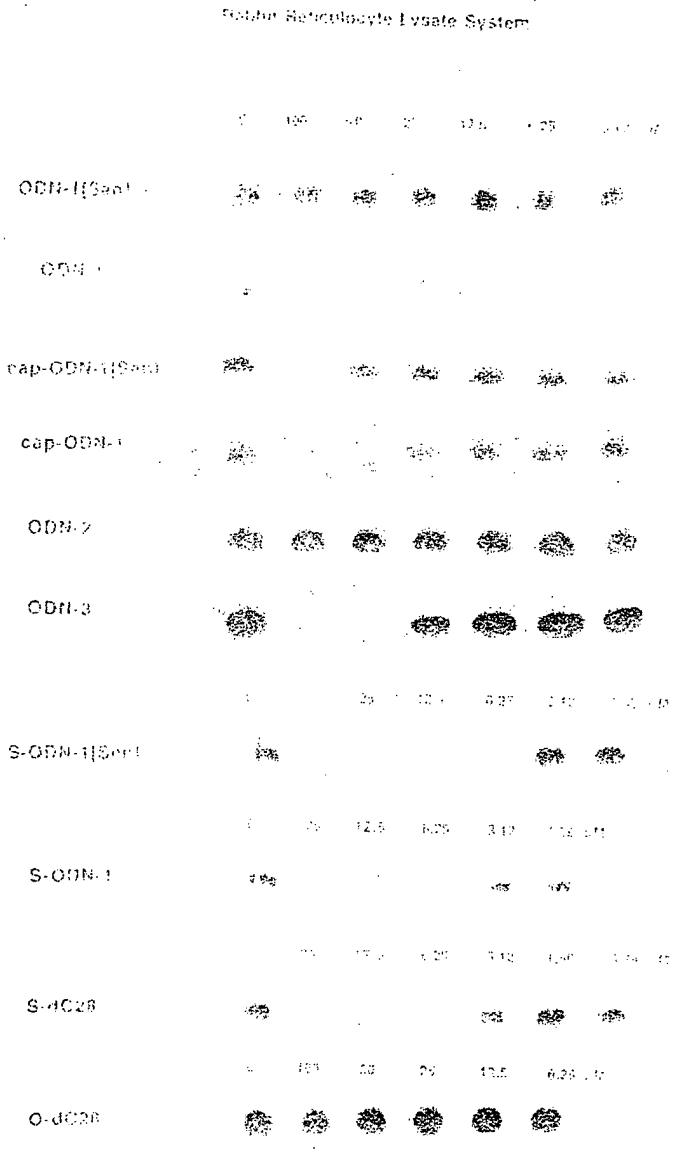


Figure 5 : Translation of rabbit globin mRNA in the reticulocyte lysate system in the presence of various oligomers (see legend of figure 4). C=control (no added oligomer). Numerals above each lane are the concentration of added oligomer (in µM or in nM). Incubation time was 90 min. at 37°C.

did not inhibit β-globin synthesis at either concentration (3.2 µM or 16 µM, lanes 3 and 6). Both 17PO-β and 17PS were partially inhibitory at low concentration (lanes 7 and 8). At higher concentration (16 µM) 17PO-β selectively inhibited the production of β-globin. In

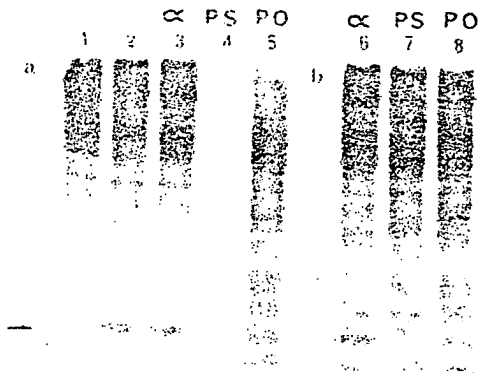


Figure 6 : Effect of various 17-mers on the synthesis of rabbit β-globin in micro-injected *Xenopus* oocytes. Autoradiograph of a 12.5% polyacrylamide-SDS gel of proteins synthesized in oocytes injected with rabbit globin mRNA in the absence (lane 2) or in the presence of 17PO-α (lanes 3 and 6), 17PS (lanes 4 and 7), or 17PO-β (lanes 5 and 8), at a concentration of 16 µM (panel a lanes 2-5) or 3.2 µM (panel b, lanes 6-8). Lane 1 corresponds to non injected oocytes. The arrow indicates the position of β-globin.

contrast, global protein synthesis was completely prevented by 17PS at this latter concentration (lane 4). This result was reminiscent to the non-specific effect induced by this oligomer in the wheat germ extract.

When the oligomers were co-injected with the message, the ability of 17PS to specifically inhibit β-globin production was greater than that of its oxygen analogue at similar concentrations (Figure 7 and Table 1). This may be due in part to the decreased sensitivity of this compound to nucleases (12). The effect of nuclease resistance on translation inhibition was better seen when a delay was introduced between injections of the oligomer and of the globin mRNA. Specific inhibition of β-globin synthesis still occurred when 17PS was injected 6 hours prior to the message although it was less efficient than upon co-injection. The concentrations leading to 50% reduction were 30 and 3 nM respectively (Figure 7b and Table 1). In contrast no effect was detected when 17PO-β was injected 6 hours prior to mRNAs even when the oligomer concentration was as high as 3 µM (Figure 7a).

In order to test a more physiological situation in which the mRNA was already engaged in translation (as is the case for endogenous RNAs from the oocyte), 17-mers were injected 6 hours after globin mRNA. Previous experiments have shown that within 6 hours after microinjection, globin mRNA is recruited into polysomes and efficiently translated. Specific inhibition of β-globin synthesis was observed in the nanomolar range with 17PS whereas 100 fold higher concentrations of 17PO-β were required for half-inhibition (Figure 7 and Table 1).

In fair agreement with what was observed in the wheat germ extract, 11PS was much less inhibitory (3 orders of magnitude) in oocytes compared to 17PS. Concentrations in the µM

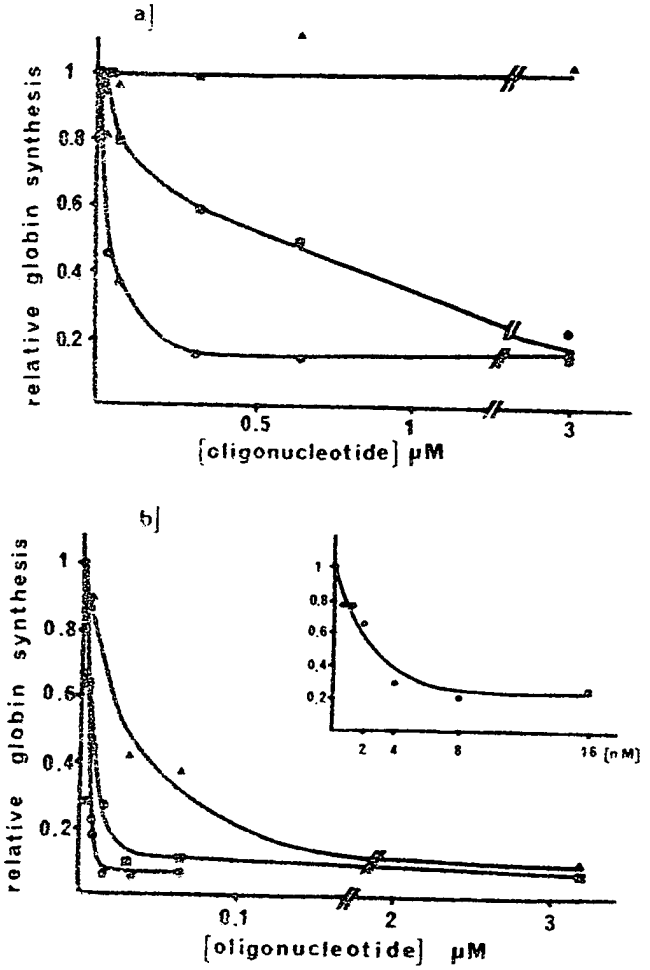


Figure 7 : Effect of 17PO-β (a) and 17PS (b) on the synthesis of rabbit β-globin in micro-injected *Xenopus* oocytes. Globin synthesis was determined from densitometer tracings of autoradiographs, relatively to the synthesis observed in the absence of added oligodeoxynucleotide. Oligonucleotides injected 6 hours prior to (▲), 6 hours after (▣) or coinjected with the mRNA (●). Inset in panel (b) is an enlargment of the curve (●) in the main figure.

range had to be used to observe 50% inhibition (Figure 3). This is probably due to the weak affinity of this oligomer for its target as indicated by a low value of the melting temperature of the DNA-RNA duplex (Table 1). It is worth mentionning that the unmodified 11PO had no effect at any concentration up to 20µM (8).

The differences in inhibition efficiencies between the various oligomers could be related to their affinity for their target or to the sensitivity to RNase-H of the hybrid they formed with β-globin mRNA. In particular it was of interest to understand why the α-oligomer had no

effect on translation. To delineate this we first investigated the binding of 17-mers to the mRNA and then the sensitivity of oligonucleotide-mRNA hybrids to RNase-H.

Thermal stability

We compared the affinity of rabbit globin mRNA for various oligonucleotides, namely 17PO-β, 17PS, 17PO-α and 16PO-α using hybridization experiments (see Materials and Methods). The two α-oligomers have the same target but whereas 16PO-α was designed to bind RNA in an antiparallel orientation, 17PO-α was synthesized to bind in a parallel orientation (Figure 1). We did not detect non-specific interactions between oligomers and the filters. All oligonucleotides but one, 16PO-α, gave a signal from thermal elution of filter-bound complexes (data not shown). Binding of 17PO-α to the immobilized mRNA indicated that this α-oligodeoxynucleotide formed a double-stranded structure with RNA in which the two chains run parallel to each other, in good agreement with a previous report (27). Assuming ΔH values are similar for all three oligonucleotides, relative affinities of 17-mer analogues for globin mRNA can be deduced from the relative temperatures Tc of half-dissociation of the complexes, given in Table 1. Even though Tc obtained with 17PO-α was lower than that of its β-homolog it was still higher than that of 17PS. Binding of 17PO-α occurred specifically to its target region of β-globin mRNA as demonstrated by a competition experiment : translation inhibition of β-globin mRNA by 17PO-β was reversed by addition of an excess of 17PO-α both in wheat germ extracts and in *Xenopus* oocytes, indicating that the two oligomers competed for binding to the same RNA sequence (data not shown). Therefore the failure to inhibit rabbit β-globin mRNA translation with the α-derivative cannot be ascribed to a weak stability of the α-oligonucleotide/mRNA hybrids.

RNase-H activity on oligodeoxynucleotide-RNA hybrids

It was shown that RNase-H, which cleaves the RNA part of RNA-DNA hybrids, amplified the antisense effect produced by oligodeoxynucleotides both in wheat germ extracts and in *Xenopus* oocytes (6-9). We therefore investigated the activity of RNase-H on RNA associated with various complementary 17-mers (17PO-β, 17PO-α and 17PS). Rabbit globin mRNA was incubated, in the presence of the oligomers, with *E.coli* RNase-H. Aliquots of the mixtures were withdrawn at various times, spotted onto nylon membranes and probed with ^{32}P end-labelled 17PO-β. As shown on figure 8 no RNase activity was detected during the time course of the experiment in the absence of added oligonucleotide. On the other hand the presence of the various 17-mers did not prevent binding of the probe. Under our experimental conditions about 85% of the β-globin mRNA was cleaved by RNase-H after a 2 h incubation in the presence of 17PO-β. Under the same conditions, 50% mRNA remained intact in the presence of 17PS, whereas no degradation was detected with 17PO-α. Therefore, although the α-oligonucleotide was bound to its target, the hybrid was not recognized as a substrate by *E.coli* RNase-H. In contrast the phosphorothioate analogue was able to induce the cleavage of the complementary RNA as previously observed with homooligomers (12).

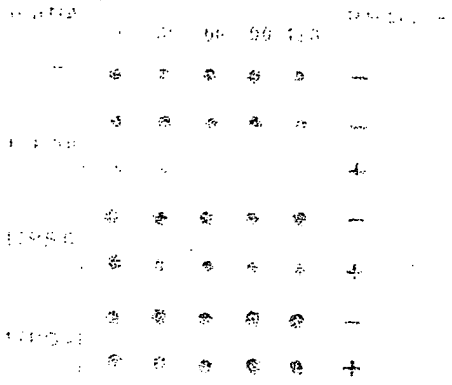


Figure 8 : Oligonucleotide-induced cleavage of rabbit β-globin mRNA by RNase-H. Dot-blot of globin mRNA incubated in the absence (T) or in the presence of 17-mers listed on the left side, in the absence (-) or in the presence of *E.coli* RNase-H (+), during the time indicated (in minutes) at the top of the autoradiograph. The blot was probed with ^{32}P-5'end-labelled 17PO-β.

DISCUSSION

As part of our studies on the use of oligodeoxynucleotides as specific inhibitors of gene expression, we have chosen to examine modified oligomers with respect to their ability to inhibit protein translation. We have focused on two modifications, phosphorothioate DNA and α-DNA which render oligonucleotides resistant to nucleases, and have compared these with the normal analogues.

The expected inhibition of globin synthesis in the reticulocyte lysate system and in the wheat germ extract was observed with normal antisense oligomers in agreement with previous reports (8, 28, 42, 43). By contrast, the situation with modified oligonucleotides was more complicated. In wheat germ extract no effect on β-globin synthesis was detected in the presence of α-oligomers (either a parallel 17-mer or an antiparallel 16-mer), targeted to the coding region of the β-globin mRNA, even at high concentrations (>30 μM). The same results were obtained in *Xenopus* oocytes: none of the two α-oligodeoxynucleotides inhibited translation of microinjected rabbit globin mRNA. This anti-β-globin sequence was synthesized in both orientations because, although it has been known for some time that α-DNA forms parallel-stranded structure with β-DNA (19-23), it was more recently reported that α-dT_8, linked to a phenanthroline-copper complex, binds to polyrA in the antiparallel orientation (25). Our studies with filter-immobilized globin mRNA showed that only the parallel 17PO-α hybridized to rabbit globin mRNA, in fair agreement with a recent work using two other mRNA species (27). Therefore it seems possible that the orientation of the two strands in an α-DNA/β-RNA hybrid depends on the base sequence of the α-oligodeoxynucleotide or that

α-oligothymidylates represent a unique case where the two strands are antiparallel rather than parallel.

The absence of inhibitory effect of the parallel 17PO-α is clearly due to the lack of activity of RNase-H on the hybrid formed with β-globin mRNA. Preliminary investigations performed with a methylphosphonate derivative of the 17-mer complementary to the region 113-129 of the β-globin mRNA support this conclusion. This phosphonate oligomer (a gift from Dr. Zon) did not prevent β-globin synthesis neither in the wheat germ cell-free system nor in micro-injected *Xenopus* oocytes at any concentration (up to 50μM). This methylphosphonate 17-mer failed to induce the degradation of the target mRNA by the *E. coli* RNase-H.

Such an RNase-H activity was previously shown to be present both in wheat germ extracts and in microinjected oocytes (8). Both the normal 17PO and its phosphorothioate analogue 17PS induced the RNaseH activity and were strongly inhibitory in both systems. But hybrids formed by rabbit β-globin with 17PS were not more susceptible to RNase-H than the ones formed with the PO analogue in contrast to what was observed with oligo(dT)'s (12). This could arise either from differences in hybrid structures or from enzyme specificity. Since RNase-H appears to be an important component in translation inhibition by complementary oligodeoxynucleotides it might be of interest to target A-rich sequences by phosphorothioate oligomers in order to take full advantage of mRNA degradation.

The experiments with phosphorothioate oligomers in microinjected *Xenopus* oocytes confirm what was surmised from cell-free experiments. The nuclease resistance properties of the S-oligonucleotides make them very active even if microinjected long before the message (Table 1). As a consequence of both DNase resistance and RNase-H activation, nearly complete inhibition could be achieved at concentrations in the low nanomolar range, i.e. at a stoichiometry of about one oligonucleotide per four mRNA molecules indicating a catalytic effect for this oligonucleotide. Results obtained from competition experiments between 17PO-α on the one hand and 17PO-β or 17PS on the other hand are also relevant : inhibition of β-globin translation by either of the latter two oligomers can be reduced by co-injection of 17PO-α. But the concentration of the 17PO-α competitor required to get a 50% reduction of the inhibitory effect in the presence of 17PO-β was lower than that in the presence of 17PS (data not shown). This could be ascribed to the intracellular degradation of 17PO-β by DNases which results in a decreasing concentration of active antimessenger during the time course of the translation experiment. These conclusions with respect to inhibition of translation are summarized in Table 2.

However there are still unanswered questions. For example 17PS binds more weakly to globin RNA than 17PO (Table 1) and it is less active at inducing cleavage of mRNA by *E. coli* RNase-H (Figure 8). In wheat germ extracts 17PO and 17PS are equally active at inhibiting β-globin synthesis. In microinjected oocytes 17PS is about ten times more active than in the

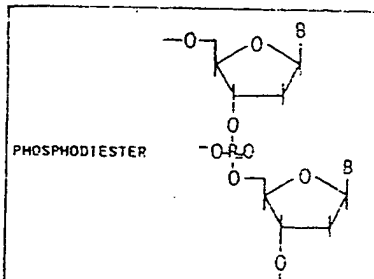
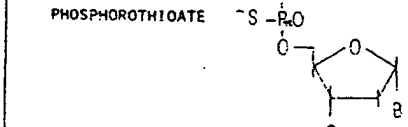
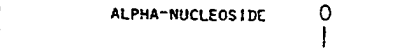
		RESISTANCE TO DNASES	ACTIVATION OF RNASE H	INHIBITION OF TRANSLATION
PHOSPHODIESTER		NO	YES	YES
PHOSPHOROTHIOATE		YES	YES	YES
ALPHA-NUCLEOSIDE		YES	NO	NO

Table 2 : Properties of unmodified, phosphorothioate and alpha-antisense oligodeoxynucleotides.

cell-free system whereas 17PO has quite similar activity. Therefore there might be other factors than nuclease resistance, hybrid stability and RNase-H susceptibility which are playing a role in the efficacy of oligodeoxynucleotides at inhibiting protein synthesis. One such factor could be different compartmentalization of the two oligomers inside oocytes. The kinetics of RNase-H cleavage could also be different in the two systems.

At least at low concentrations (<1μM for 17PS) phosphorothioate analogues fulfill the criteria of anti-sense specificity: 17PS inhibits the synthesis of its target only, i.e., β-globin. But in this paper, we have demonstrated that these derivatives may be non-sequence specific inhibitors of protein synthesis if the concentration is not optimized. We have shown that this effect is highly concentration and length dependent: in wheat germ extract a 5-mer PS was not toxic at 100 μM but a 15-mer containing two blocks of 5 contiguous phosphorothioates behaved like a 10-mer PS. In contrast, in the reticulocyte lysate, a 15-mer phosphodiester capped with phosphorothioate units at both ends exhibited specific behavior close to that of the all-phosphodiester analogue. The results presented in this paper can be discussed with respect to the effects of S-oligomers in other systems. For instance studies on HIV replication and protein expression have revealed two distinct mechanisms of inhibition by phosphorothioate oligodeoxynucleotides: one sequence (antisense) specific, the other non-sequence specific (15, 44, Matsukura et al., unpublished results). Kinetic studies of cellular uptake of fluorescent oligomers have identified an 80kD protein as a possible cell surface receptor for oligonucleotides and related substances (45, 46). In addition, Zhang et al. (unpublished results), have shown that the 80kD protein binds phosphorothioate with greater avidity than normal DNA. A similar observation was noted for HIV reverse transcriptase, which also binds phosphorothioate DNA with higher affinity than its oxygen congener (50). In subsequent

experiments, it has been shown that although both phosphorothioate oligomers and their normal counterparts bind to ribosomes, the former is not displaceable (Stein and Neckers, unpublished results). It is thus of interest to note that when 17PS was injected into *Xenopus* oocytes in high enough concentration (16 μM), total protein synthesis was abolished and the oocytes exhibited altered pigmentation and then underwent extensive cytolysis. These observations may account for the non-specific cellular cytotoxicity observed when cells are exposed to concentrations of phosphorothioate DNA above 25-50 μM for extended times. However it is worth noting that in *Xenopus* oocytes specific translation inhibition by a 17-mer was achieved in the nanomolar range i.e. at concentrations three orders of magnitude lower than that at which toxic effects took place.

From the standpoint of antisense strategy, phosphorothioate DNA appears to be one promising member of the class of modified oligonucleotides. These compounds will very likely be alternative tools to unmodified derivatives in the field of developmental biology and in drug research. These compounds could allow one to specifically ablate the expression of a gene in cells at very low concentrations of antisense molecules, without detrimental effect or interference with the intracellular nucleotide pool, in contrast to recent reports in which unmodified phosphodiester oligonuclectides have been used (47-49).

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Abbreviations:

ODN, oligodeoxynucleotide; PO, phosphodiester oligomer; PS, phosphorothioate oligomer; α, alpha oligomer.

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RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds

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ABSTRACT

Three types of 14-mer oligonucleotides were hybridized to human β-globin pre-mRNA and the resultant duplexes were tested for susceptibility to cleavage by RNase H from E.coli or from HeLa cell nuclear extract. The oligonucleotides contained normal deoxynucleotides, phosphorothioate analogs alternating with normal deoxynucleotides, or one to six methylphosphonate deoxynucleosides. Duplexes formed with deoxyoligonucleotides or phosphorothioate analogs were susceptible to cleavage by RNase H from both sources, whereas a duplex formed with an oligonucleotide containing six methylphosphonate deoxynucleosides alternating with normal deoxynucleotides was resistant. Susceptibility to cleavage by RNase H increased parallel to a reduction in the number of methylphosphonate residues in the oligonucleotide.

Stability of the oligonucleotides in the nuclear extract from HeLa cells was also tested. Whereas deoxyoligonucleotides were rapidly degraded, oligonucleotides containing alternating methylphosphonate residues remained unchanged after 70 minutes of incubation. Other oligonucleotides exhibited intermediate stability.

INTRODUCTION

Antisense oligonucleotides are increasingly used as modulators of cellular and viral gene expression (see ref. 1−4 for review). Three classes of oligonucleotides have been used in recent investigations: antisense deoxyoligonucleotides (D-oligos), their modified counterparts and antisense RNA. All three classes have been effective in inhibiting expression of specific genes. For example, in their pioneering work Zamecnik and Stephenson (5) showed that D-oligos complementary to a segment of reiterated terminal sequence of Rous sarcoma virus inhibit viral replication. More recently phosphorothioate deoxyoligonucleosides (S-oligos, developed by Eckstein and coworkers, see ref. 6 for review), methylphosphonate deoxyoligonucleosides (MP-oligos, developed by Miller, Ts'o and coworkers, reviewed in ref. 7) as well as D-oligos have been shown to inhibit replication of the human immunodefficiency virus when they were complementary to essential viral sequences (8−11). Other modified oligonucleotides that inhibit expression of specific genes include phosphoroamidate oligonucleosides (9), α-oligonucleotides (12,13), and polylysine (14), psoralen (15) and acridine conjugated oligonucleotides (16,17). The discovery that the expression of some procaryotic genes is controlled in vivo by endogenous antisense RNA (reviewed in 18 and 19), and that expression of thymidine kinase can be inhibited by antisense RNA transcribed from a recombinant expression vector (20,21) showed that antisense RNA may also be useful in inhibiting the expression of specific genes. This early work led to a number of subsequent studies extensively reviewed in ref. 18.

Most of the reports discussed above focused on the final effect of antisense oligonucleotides on gene expression without detailed studies of the mechanism of inhibition. The two most likely mechanisms of inhibition appear to be 1) direct blocking in pre-mRNA and/or mRNA of sequences important for processing or translation and 2) degradation of the RNA transcript by RNase H at the site of oligonucleotide binding. RNase H cleaves the RNA component of RNA:DNA hybrids and is abundant in the cytoplasm and nucleus of a large number of organisms (22). Recent reports show that cleavage of RNA:DNA duplexes by RNase H was predominantly responsible for the inhibitory activity of D-oligos in several experimental systems (23–25). However, only limited data are available regarding the mechanism of action of modified oligonucleotides (3,26,27). In addition, discrepancies exist concerning the effects of antisense molecules. For example, antisense MP-oligos were found inhibitory in some but not all systems (10,11, 26,28,29) and a 1000 fold excess of antisense RNA did not inhibit the activity of chloramphenicol acetyl transferase expressed in transfected CV1 cells (30). These observations suggest that more detailed investigations are needed to discern the mechanism of inhibition by antisense oligonucleotides.

Since there are indications that RNA:MP-oligo duplexes are resistant to RNase H (3,26) we decided to study in more detail the effect of incorporation of methylphosphonate deoxynucleosides into D-oligos on the susceptibility of pre-mRNA:DNA duplexes to RNase H cleavage. To this end we have used a series of 14-mer oligonucleotides substituted with one to six methylphosphonate deoxynucleosides. For comparison, we have also tested a 14-mer D-oligo and a 14-mer S-oligo containing alternating deoxynucleotides and phosphorothioate analogs. We found that D-oligo and an alternating S-oligo form duplexes with pre-mRNA that are cleaved by E.coli RNase H and by the RNase H present in a crude nuclear extract from HeLa cells. In contrast, RNA in duplexes formed with an MP-oligo containing six methylphosphonate deoxynucleosides alternating with deoxynucleotides is resistant to cleavage by RNase H from both sources but becomes susceptible if the number of methylphosphonate deoxynucleosides in the oligonucleotide is decreased.

MATERIALS AND METHODS

Oligonucleotide synthesis. Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems) using standard β-cyanoethyl cycles (31). Materials for synthesis were supplied by Applied Biosystems or American Bionetics. D-oligos were deblocked and cleaved from the column with following the Applied Biosystems protocol. S-oligos were obtained in a purified form from Dr. Scott Eadie (Applied Biosystems). MP-oligos were cleaved from the solid support column by ethylenediamine:absolute ethanol (1:1 v/v) treatment at 55°C for 55 minutes, eluted with absolute ethanol followed by ethanol-water (1:1 v/v), lyophilized and resuspended in water. $O.D._{260}$ was measured for each sample and the oligonucleotides were used in this form for all experiments. When necessary, all oligonucleotides were end labeled with [^{32}P] γ-labeled ATP using T4 polynucleotide kinase in 100 mM Tris pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine, 0.2 mM EDTA at 37°C for 30 minutes as recommended by the supplier (New England Biolabs).

Pre-mRNA transcription. The DNA plasmid containing the human β-globin gene cloned under the control of the SP6 promoter (pSP64HbΔ6, see ref. 32), was digested with the restriction endonuclease Bam HI and transcribed and capped in vitro as described (33) using SP6 polymerase and [^{32}P] labeled GTP (New England Nuclear). This produced a human β-globin pre-mRNA truncated at the 3′ end of the second exon.

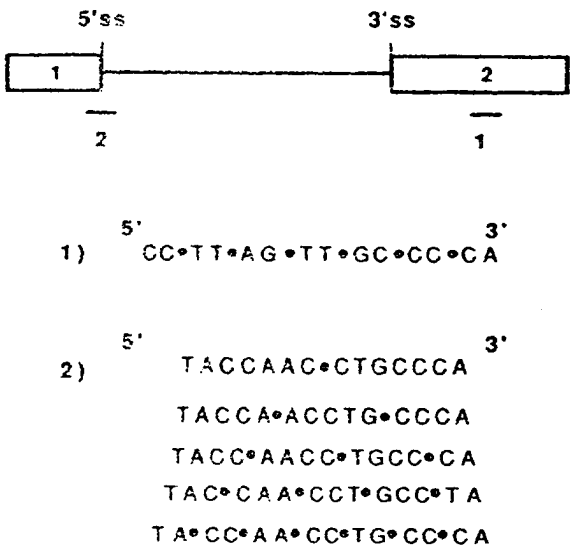

Figure 1. Target RNA and antisense oligonucleotides. The structure of the truncated human β-globin pre-mRNA used for hybridization with the oligonucleotides is shown. Exons (boxes) intron (thin line) and splice sites (5' and 3' ss) are indicated. The transcript is terminated at a Bam HI site close to the 3' end of the second exon. The positions where oligos #1 and #2 hybridize to the pre-mRNA as well as the sequence of the oligos are also shown. Dots in the oligonucleotide sequence show the position of the methylphosphonate or phosphorothioate internucleotide bonds in MP- and S-oligos. Oligo #1 was used as a normal deoxyolignucleotide (D.1), an alternating phosphorothioate (S.1) and an alternating methylphosphonate (MP.1). Oligo #2 was used as a deoxyoligonucleotide (D.2) or containing an increasing number of methylphosphonate residues per molecule (1MP.2–6MP.2).

RNase H cleavage. E.coli RNase H was obtained from Bethesda Research Laboratories. Nuclear extract from HeLa cells (32,34) was used as a source of eucaryotic RNase H. Oligonucleotides (25 pmoles) and [^{32}P] labeled pre-mRNA (10 pmoles) were hybridized in vitro at 40°C for 10 minutes in 10 μl of the following reaction mixture: for cleavage by E. coli RNase H, the hybridization was performed in 130 mM ammonium chloride; for cleavage by RNase H from HeLa cell nuclear extract, the hybridization mixture contained 12.5 mM ATP, 8.25 mM $MgCl_2$, 50 mM creatine phosphate and 6.5% polyvinyl alcohol. Following hybridization, for the E.coli enzyme, the reaction was performed at 37°C for 30 minutes in a total volume of 20 μl containing 130 mM ammonium chloride, 10 mM Tris pH 7.5, 10 mM magnesium acetate, 5% sucrose and 1μl of RNase H. For the HeLa enzyme, 15 μl of the nuclear extract was added and the reaction was performed at 30°C for 15 minutes. The extract contributed several buffer components so that the final concentrations of the reagents were 5 mM ATP, 3.3 mM $MgCl_2$, 20 mM creatine phosphate, 2.6% polyvinyl alcohol, 12.8 mM HEPES, pH 7.9, 14% glycerol, 60 mM KCl, 0.12 mM EDTA and 0.7 mM DTT, i.e., standard conditions for in vitro splicing of pre-mRNA (34). RNase H cleavage products were analyzed by electrophoresis on a 5% polyacrylamide followed by autoradiography.

Primer extension. Primer extension assay was performed in a 10 μl reaction containing 100 mM Tris pH 7.5, 100 mM $MgCl_2$, 200 mM NaCl, 25 pmoles of oligonucleotide, 10 pmoles of [^{32}P]-pre-mRNA, 5 μCi of [^{32}P]-α-labeled dATP, 2.5 mM deoxynucleotide triphosphates and 1 μl of AMV reverse transcriptase (Life Sciences). The primer extension

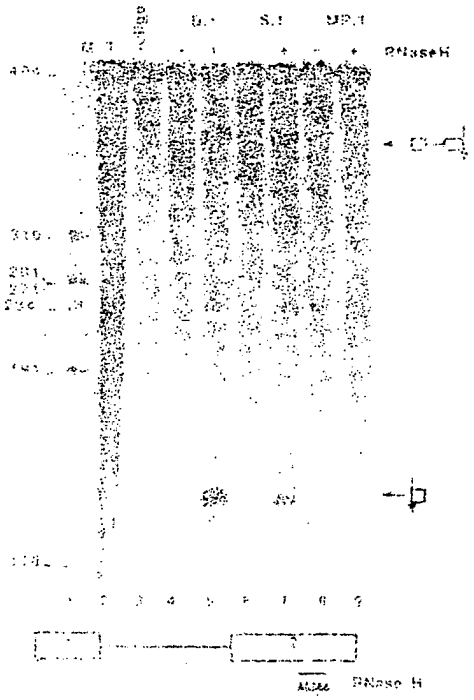

Figure 2 A. Cleavage of pre-mRNA in duplexes with D-oligo, S-oligo, or MP-oligo by RNase H from E.coli. [^{32}P]-pre-mRNA was hybridized with oligonucleotide # 1 (see Fig. 1) in the form of D-oligo (D.1, lanes 4 and 5), alternating S-oligo (S.1, lanes 6 and 7)), or alternating MP-oligo (MP.1, lanes 8 and 9) and incubated without (−) or with (+) RNase H from E. coli. The resulting RNA products were separated on a 5% polyacrylamide sequencing gel. A schematic structure of RNase H cleavage products is shown at right. Below the figure, the structure of the pre-mRNA, the position of binding of oligonucleotide # 1, and cleavage by RNase H are shown. In this and subsequent figures, M (lane 1) denotes size markers (Hae III digest of ΦX174 in this figure and products of β-globin pre-mRNA splicing in the following figures), T (lane 2) denotes untreated RNA transcript (approximately 3 times the amount of radioactive RNA was loaded in this lane), -oligo (lane 3) denotes pre-mRNA mock hybridized and incubated with RNase H in the absence of oligonucleotide.

products were electrophoresed and autoradiographed as above.

Stability of oligonucleotides. Oligonucleotides, 5′-end labeled with [^{32}P], were incubated in the HeLa cell nuclear extract for 0−70 minutes in the same buffer conditions as described above and subsequently analyzed on a 20% polyacrylamide sequencing gel. The gels were autoradiographed and the amount of intact material and degradation products was quantitated by densitometry. As a measure of degradation a ratio of the amount of the intact 14-mer to a smallest degradation product, the mononucleotide, was calculated and plotted in figures 3 and 7. This way of calculation was carried out to compensate for the activity of phosphatases, which might be present in the nuclear extract and could gradually remove the radioactive label.

RESULTS

A capped fragment of human β-globin pre-mRNA (Fig. 1), obtained by transcription in vitro (see Materials and Methods), was hybridized to three types of 14-mer oligonucleotides that included a normal deoxynucleotide (D-oligo), a phosphorothioate analog (S-oligo),

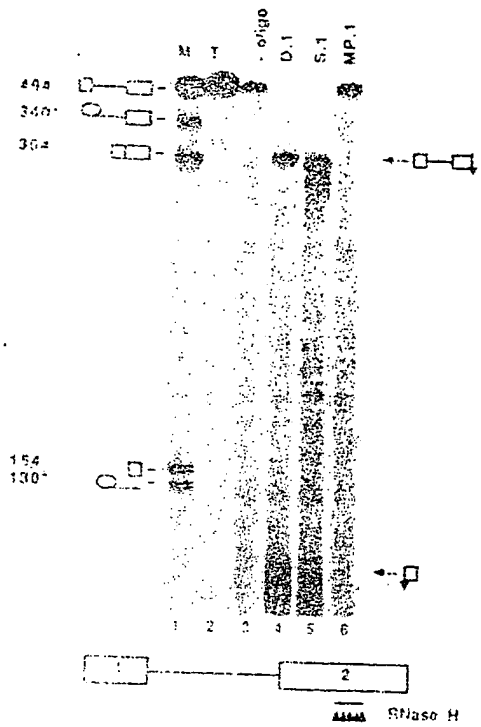

Figure 2B. Cleavage of pre-mRNA in duplexes with D-oligo, S-oligo or MP-oligo by RNase H from HeLa cell extract. The same [^{32}P]-pre-mRNA:oligo duplexes as in A were treated with RNase H from HeLa cell nuclear extract (lanes 4, 5, and 6). RNA products were analyzed as in A and the structure and position of the RNase H cleavage products are shown on the right. In lane 1, pre-mRNA was spliced in vitro and the resulting products used as size markers. The size and structure of the splicing products is shown at left. Asterisk indicates aberrant migration on the gel of the splicing intermediates containing lariat. Below the figure, a diagram of the RNase H site of cleavage is shown.

and a series of oligonucleotides containing an increasing number of methylphosphonate deoxynucleosides (MP-oligos). The D-oligo, the S-oligo and one of the MP-oligos were complementary to nucleotides 360–373 in the second exon of human β-globin pre-mRNA (Fig. 1, oligo 1). The S-oligo and the MP-oligo contained alternating phosphodiester and modified internucleotide bonds. An additional series of MP-oligos, which contained from one to six methylphosphonate deoxynucleosides positioned in the oligonucleotides as shown in Fig. 1, was complementary to the 5′ splice site, at nucleotides 148–161 (Fig. 1, oligo 2). Substitution of six methylphosphonate deoxynucleosides resulted in an MP-oligo containing alternating methylphosphonate and phosphodiester internucleotide bonds. The duplexes formed with these oligonucleotides were tested for their susceptibility to hydrolysis by RNase H.

The pre-mRNA hybridized with the normal D-oligo, complementary to the second exon (Fig. 1, oligo 1), was incubated with E.coli RNase H and the RNA isolated from the reaction was analyzed on a 5% polyacrylamide sequencing gel. Part of the RNA was cleaved into two fragments of approximately 360 and 120 nucleotides (Fig. 2A, lane 5). Longer incubation with a larger amount of enzyme led to complete cleavage of the RNA into the same two fragments (results not shown, see also Fig. 5, lane 9). The larger cleavage product

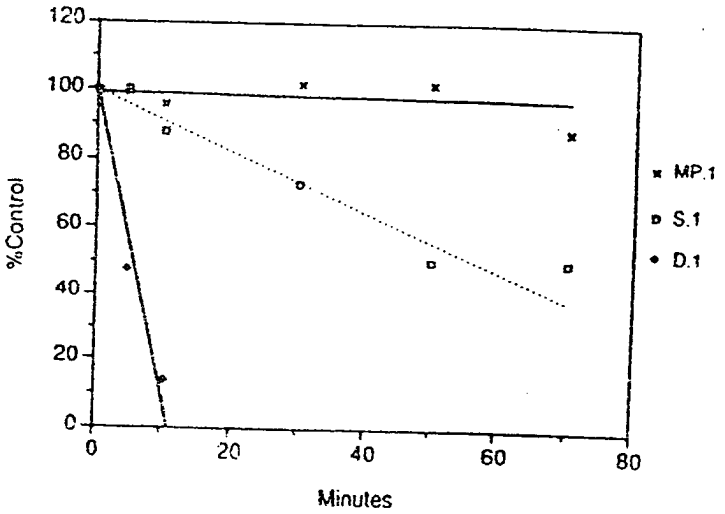

Figure 3. Stability of oligonucleotide # 1 in a HeLa cell nuclear extract. D-oligo (D.1), S-oligo (S.1) and MP-oligo (MP.1) forms of oligonucleotide # 1 were labeled with [^{32}P] using T4 polynucleotide kinase and incubated from 0–70 minutes in a HeLa cell nuclear extract. The oligonucleotides were separated on a 20% polyacrylamide sequencing gel and the amount of intact material and degradation products was quantitated by densitometry of autoradiograms. Extent of degradation was calculated as a ratio of the amount of the intact 14-mer to the smallest degradation product, the mononucleotide.

represents a capped 5′ fragment of the pre-mRNA located upstream from the binding site of the oligonucleotide while the smaller fragment represents a 3′ part of the RNA transcript. The mobility of the fragments on the gel is in agreement with their size predicted from the RNA sequence data. The cleavage reaction required both the oligonucleotide and the enzyme, since the RNA remained intact in control samples missing either of these components, (Fig. 2A, lanes 1 and 2, respectively). Similarly, oligonucleotide and enzyme-dependent cleavage by RNase H was also observed for a duplex containing the alternating S-oligo (Fig. 2A, lane 7) although densitometry of the film showed that the yield of the generated fragments was approximately 20 % lower than that for the D-oligo containing duplex (compare lanes 5 and 7 in Fig. 2A). In contrast, RNA hybridized under the same conditions with alternating MP-oligo was resistant to cleavage by RNase H (Fig. 2A, lane 9).

To determine whether eucaryotic RNase H would cleave these duplexes, the same samples were incubated with a nuclear extract from HeLa cells known to contain high levels of RNase H activity (32). The crude extract was used because it more closely resembles the intracellular conditions to which the duplexes would be exposed in vivo. Similarly as in the previous experiment, pre-mRNA in duplexes with either D-oligo or S-oligo was hydrolyzed by RNase H in the extract (Fig. 2B, lanes 4 and 5, respectively) whereas the pre-mRNA hybridized with MP-oligo remained intact (Fig. 2B, lane 6). The 360 nucleotide fragment generated by RNase H cleavage was stable in the extract and is clearly visible on a gel but the shorter 3′ fragment, which was not capped, was largely degraded. The instability of the latter fragment is in agreement with the observations that the nuclear extract from HeLa cells contains a 5′ to 3′ exonuclease activity which is inhibited by the cap structure at the 5′ end of the pre-mRNA transcript (32 and our unpublished observations).

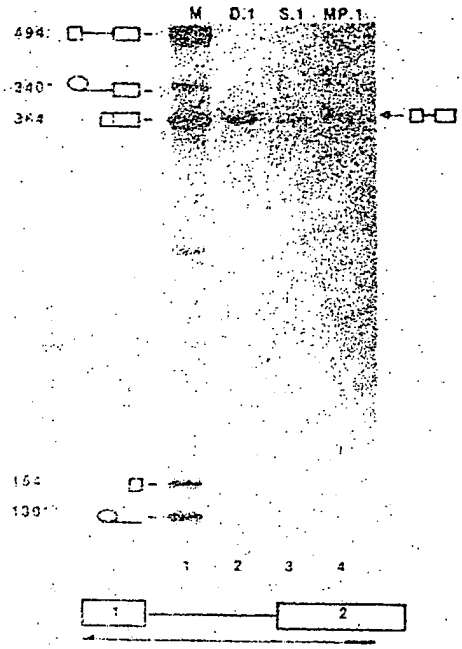

Figure 4. Primer extension analysis of oligonucleotide analogues. D-oligo, S-oligo, and MP-oligo forms of oligonucleotide # 1 were hybridized to the pre-mRNA, extended with AMV reverse transcriptase and the products separated on a 5% polyacrylamide gel. The structures and positions of the primer extension products for D-oligo (D.1, lane 2), S-oligo (S.1, lane 3), and MP-oligo (MP.1, lane 4) are shown on the right. A diagram of the primer extension reaction is shown at the bottom.

The above results showed that pre-mRNA hybridized with D-oligo and an alternating S-oligo formed duplexes that were substrates for RNase H. However, the lack of cleavage of the pre-mRNA hybridized with an alternating MP-oligo either by E. coli RNase H or by the HeLa cell nuclear extract could have several possible explanations. Although it is likely that the pre-mRNA:MP-oligo duplex may not be a substrate for RNase H, it is also possible that 1) the alternating MP-oligo is rapidly degraded, especially in the crude nuclear extract, 2) it does not hybridize to pre-mRNA under the conditions of the experiment, or 3) it directly inhibits RNase H. A series of experiments have been performed to distinguish between these possibilities.

To test the stability of the MP-oligo in the extract, the oligo was labeled with [^{32}P] using T4 polynucleotide kinase, incubated in the nuclear extract and analyzed on a 20% polyacrylamide sequencing gel. The autoradiograms of the gel were quantitated by densitometry (see Materials and Methods). For comparison, the stabilities of D-oligo and S-oligo were also tested. Of the three oligonucleotides, MP-oligo was the most stable and remained essentially intact for at least 70 minutes of incubation. Degradation of S-oligo was also slow, with approximately 50% of the material remaining at the end of incubation whereas D-oligo was almost completely degraded after 10 minutes (Fig. 3).

To determine whether the MP-oligo is able to hybridize to the pre-mRNA, we tested it in a primer extension assay (Fig. 4). All three oligonucleotides, including MP-oligo, generated extension products of the expected length (374 nucleotides) indicating that,

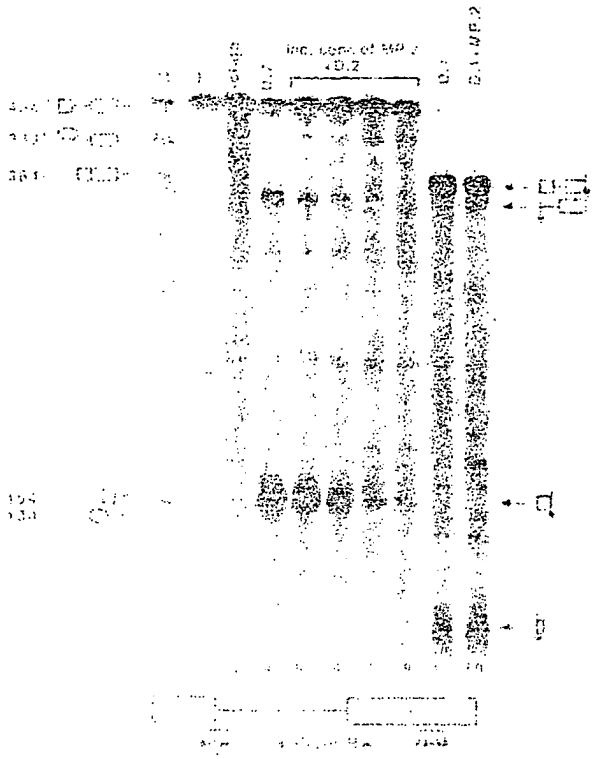

Figure 5. Competition assay using D-oligo #2, alternating MP-oligo #2 and D-oligo #1. 10 pmoles of [^{32}P]-labeled pre-mRNA was hybridized with 0, 0.002, 0.02, 0.2 or 1O.D.$_{260}$ of MP-oligo #2 followed by hybridization with 0.002 O.D.$_{260}$ of D-oligo #2 and treatment with RNase H from E. coli (lanes 4–8, respectively). The position and structure of the RNase H cleavage products after separation on a 5% polyacrylamide gel are shown by the middle two diagrams on the right. To test for direct inhibition of RNase H by MP-oligo, pre-mRNA was hybridized without (lane 9) or with (lane 10) MP-oligo #2 followed by hybridization with D-oligo # 1 and treatment with RNase H from E. coli. The position and structure of these RNase H cleavage products are shown by the top and bottom diagrams on the right. Below the figure is a schematic representation of the RNase H cleavage site for the oligonucleotides used above.

similarly to D-oligo and S-oligo, MP-oligo formed a duplex with the pre-mRNA. Additional faint bands seen in lane 2 result from premature termination of reverse transcription. The lower yield of extension products from the S-oligo and MP-oligo duplexes as compared to the D-oligo duplex is probably due to the lower efficiency of the AMV reverse transcriptase with the modified primers, as previously observed (35). Since S-oligo and MP-oligo yielded similar amounts of extension product, the resistance of the RNA:MP-oligo duplex to hydrolysis by RNase H (Fig. 2A, lane 9 and Fig. 2B, lane 6) was not due to the lack of hybridization of this oligonucleotide to the pre-mRNA.

To obtain additional evidence that the interaction of MP-oligo with the pre-mRNA is sequence specific and to show that the MP-oligo does not directly inhibit RNase H, we carried out a competition experiment with different D- and MP-oligos. The pre-mRNA was hybridized to increasing concentrations of the alternating MP-oligo complementary to the 5′ splice site (see Fig. 1B, oligo 2) followed by incubation with a normal D-oligo

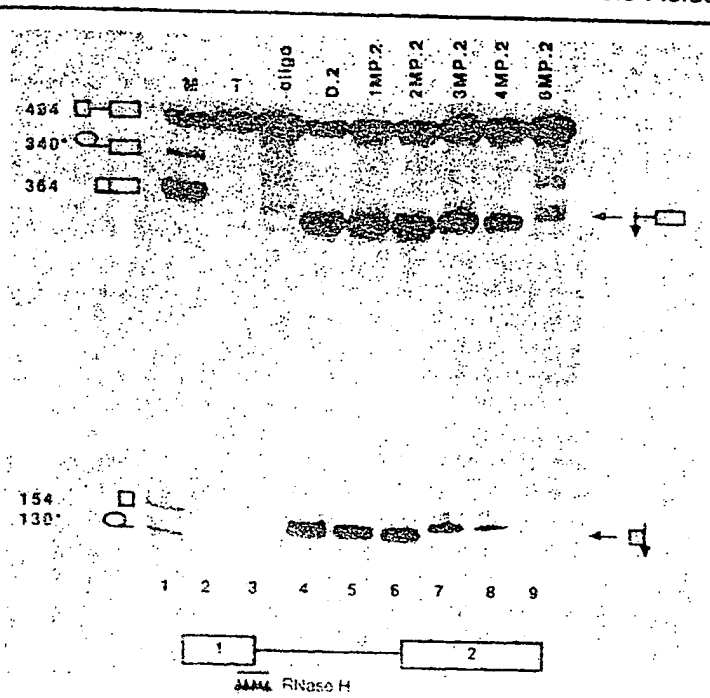

Figure 6. Effect of the number of MP-deoxynucleosides in oligonucleotide #2 on the susceptibility of RNA:MP-oligo duplexes to RNase H cleavage. Pre-mRNA:oligo duplexes were formed and treated with E.coli RNase H as described in Materials and Methods and analyzed on a 5% polyacrylamide sequencing gel. Size markers and controls (lanes 1–3) are as described in Fig. 2A. Lane 4, RNase H cleavage of a duplex with D-oligo #2 (D.2). Lanes 5–9, RNase H cleavage of duplexes with MP-oligos containing one to six MP-deoxynucleosides, respectively. Diagrams are as described in Fig 2A.

of the same sequence. The duplexes were then treated with RNase H in the nuclear extract as described above. As expected, in the absence of MP-oligo, the pre-mRNA was cleaved into two RNase H cleavage products, approximately 330 and 150 nucleotides long (Fig. 5, lane 4). Cleavage with RNase H was progressively inhibited by increasing amounts of MP-oligo as indicated by the disappearance of the cleavage products in lanes 5–8. Other bands visible in these lanes represent unspecific degradation products since they are also present in the control sample incubated without either oligonucleotide (Fig. 5, lane 3). To ascertain that the MP-oligo did not inhibit RNase H directly, the D-oligo complementary to the exon sequence (Fig. 1, oligo 1) was hybridized to the pre-mRNA with or without the alternating MP-oligo complementary to the 5′ splice site (Fig.1, oligo 2). When the duplexes were incubated with the nuclear extract, RNase H cleaved the pre-mRNA:D-oligo duplex and was not inhibited in the presence of the MP-oligo (Fig. 5, lanes 9 and 10).

We conclude from the experiments that 1) the MP-oligo did not have any direct inhibitory effect on RNase H; 2) the increasing resistance of duplexes seen in lanes 5–8 must be due to competition between D-oligo and MP-oligo for the same sequence in the pre-mRNA; 3) the MP-oligo formed a sequence specific duplex with the pre-mRNA which was resistant to RNase H.

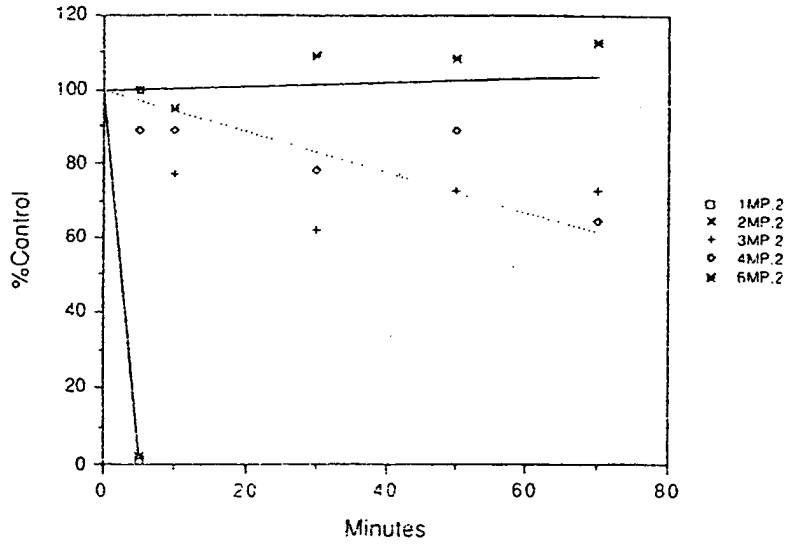

Figure 7. Effect of the number of MP-deoxynucleosides in oligonucleotide #2 on the stability of the MP-oligos in a nuclear extract from HeLa cells. MP-oligos containing one to six MP-deoxynucleosides (1MP.2–6MP.2, respectively) were incubated in the nuclear extract from HeLa cells and analyzed as described in Fig.3.

Since the above results showed that duplexes between pre-mRNA and alternating MP-oligo are resistant to RNase H we wanted determine the minimum number of methylphosphonate deoxynucleosides required to confer resistance to a duplex molecule. We have synthesized a series of 14-mer oligonucleotides, containing from one to six methylphosphonate deoxynucleosides positioned in the oligonucleotides as shown in Fig. 1 (Fig. 1, oligo 2). To ascertain that the resistance to RNase H observed above is not due to a particular sequence or secondary structure at the oligonucleotide binding site in the second exon, this series was made complementary to a different region of the pre-mRNA, at the 5′ splice site. These MP-oligos were hybridized to the pre-mRNA and the duplexes were subjected to hydrolysis by E. coli RNase H.

RNA hybridized to MP-oligos containing one or two methylphosphonate deoxynucleosides was cleaved by RNase H almost as easily as that in the control duplex with D-oligo (Fig. 6, compare lane 4 with lanes 5 and 6). RNA in duplexes with MP-oligos which contained three, four and six methylphosphonate deoxynucleosides, i.e., in which methylphosphonate bonds were separated by three, two or one phosphodiester bond (see Fig.1, oligo 2), was increasingly resistant to cleavage by the enzyme (Fig. 6, lanes 7–9, respectively). Interestingly, the resistance of these duplexes to RNase H hydrolysis paralleled the stability of the corresponding MP-oligos in the nuclear extract . MP-oligos containing one or two methylphosphonate deoxynucleosides were degraded rapidly and the stability of the oligonucleotides increased with an increased number of methylphosphonate deoxynucleosides in the molecule (Fig. 7)

DISCUSSION

Results presented above show that D-oligo, S-oligo and MP-oligos hybridize to pre-mRNA in a sequence specific manner. Duplexes formed with the first two types of oligonucleotides are susceptible to cleavage by RNase H from E. coli and from HeLa cells. This agrees

with the results of Stein et al. (27) on cleavage of polyA:phosphorothioate oligo dT duplexes by RNase H from E. coli and with the reports of other groups on inhibition of translation by RNase H cleavage of D-oligo containing duplexes (23–25). In contrast, duplexes formed with alternating MP-oligos are not substrates for RNase H from E.coli or HeLa cells. Their resistance to the enzyme decreases with the decrease in the number of methylphosphonate deoxynucleosides in the MP-oligo. Our results also show that the presence of phosphorothioate and methylphosphonate deoxynucleosides promotes the stability of the oligonucleotides in the nuclear extract from HeLa cells.

It has been shown by a number of investigators that D-oligos, S-oligos and MP-oligos can be used in vivo to inhibit expression of a specific gene in a sequence specific manner (reviewed in 1–4). The results presented here suggest different mechanisms of inhibition by different classes of oligonucleotides. Whereas D-oligos and S-oligos probably lead to the degradation of target RNA transcript by RNase H, MP-oligos containing only methylphosphonate deoxynucleosides or alternating MP-oligos exert their effect in a different manner, most likely by interfering with the accessibility of RNA to factors essential for processing, transport or translation. In most experiments, designed to eliminate RNA viruses, viral transcripts, or the inappropriate expression of endogenous RNA, the cleavage of an RNA:DNA hybrid is desired. In some situations, however, the lack of cleavage of the RNA can be advantageous. For example, the ability to block specific sequences in intact pre-mRNA may be very useful in studies of the interactions of the splicing factors with the pre-mRNA substrate. In fact, our recent experiments show that alternating MP-oligo complementary to the 5′ splice site of the β-globin pre-mRNA will hybridize to the pre-mRNA substrate without degrading it and will inhibit splicing in the nuclear extract from HeLa cells (Furdon and Kole, unpublished).

Our results with MP-oligos containing an increasing number of methylphosphonate residues suggest that the properties of the MP-oligos may be manipulated to achieve a desired effect. Introduction of a few methylphosphonate deoxynucleosides will not inhibit the activity of RNase H but will markedly increase the stability of the resulting MP-oligos. Thus, if degradation of the target RNA is the goal of the experiment such MP-oligos should be more effective than normal D-oligos. Conversely, for blocking of specific sequences in intact RNA , MP-oligos made exclusively with methylphosphonate deoxynucleosides or with alternating methylphosphonate and normal deoxynucleotides should be preferable. The alternating MP-oligos have a higher negative charge than fully modified MP-oligos allowing for easier handling and purification on polyacrylamide gels. In addition, in alternating MP-oligos the number of diastereoisomers generated during chemical synthesis (36) is drastically reduced which may increase the effective concentration of the molecules able to hybridize to target RNA. Finally, MP-oligos that contain two phosphodiester bonds surrounded by stretches of methylphosphonate deoxynucleosides can be used as tools for precise cleavage of the target RNA by RNase H.

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